A Study of Kanglaite Injection Treated Human Erythroleukemia K562 Cells with TUNEL and Annexin V Labeling Technique - Flow Cytometric Scan Methods

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Abstract

Purpose: To study the mechanism underlying the direct killing effects of Kanglaite Injection (KLT) on tumor cells and its role in chemotherapy. Method: Observing the apoptosis of human tumor cells induced by KLT with Terminal Deoxynucleotidyl Transferase (TUNEL) and Annexin V Labeling techniques. Result: KLT had evident proliferation-inhibiting action for human erythroleukemia cells and solid tumor cells. Apoptosis and necrosis with damaged cell membrane in colon cancer SW1116 cells and erythroleukemia K562 cells induced by 10 μl/ml KLT were observed with TUNEL and Annexin V Labeling methods. Conclusion: The key mechanism of KLT anticancer effects might be interpreted by the apoptosis and necrosis of tumor cells it induced.

Carcinogenesis is related with uncontrolled proliferation and block of apoptosis of tumor cells. Apoptosis of tumor cells is now a hot topic in oncology investigation and measures for inducing tumor cell apoptosis have emerged as a new model in malignant cancer treatment. Recently Chinese scientists have reported that treatment of acute premyelocytic leukemia (APL) with arsenical (SeO3) has obtained satisfactory results. They have demonstrated that SeO3 can induce apoptosis of APL cells and have established a successful model for apoptosis induction therapy. Pharmacological studies on KLT in vivo and in vitro have demonstrated its killing effects for many tumor cells. Recent studies showed that KLT could inhibit proliferation of tumor cells due to its action for retarding G2/M phase, decreasing cell number in S phase and reducing mitosis of cancer cells. But nothing about KLT induced apoptosis of tumor cells had ever been reported. In this study an observation was undertaken to investigate in vitro the apoptosis of human tumor cells induced by KLT in an attempt to serve as a theoretical basis for the management of cancers with KLT.

1. Materials and methods

1.1 Cell culture and drugs
Tumor cells including human gastric cancer MKN 28 cells, human colon cancer SW1116, COLO 205 and WiDr cells, human hepatic cancer 7901 cells, human erythroleukemia K562 cells and human squamous carcinoma KB cells were cultured and passaged in RPMI 1640 medium with 10% calf serum (Gibco USA) in our laboratory. KLT (10%) and emulsion were supplied by Zhejiang Kanglaite Pharmaceutical Co., Ltd. Blue tetrazolium [3 (4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide, MTT], In Situ Cell Death Detection kits (TUNEL) and Annexin-V-Fluos labeling assay agents were bought from Sigma and Boehringer Mannheim respectively.
1.2 Instruments
Flow cytometric scan (FAC Scan) was purchased from Becton Dickinson. Full automatic enzyme labeling photometer (Sigma 960) was purchased from Sigma, U.S.A.

1.3 Methyl Thiazole Tetrazolium Reduction Method (MTT Method)
Logarithmic growth phase tumor cells 1×10⁴/ml were inoculated into each well of a 96-well microculture plate. A saline control group (well) and experiment groups (wells) of different KLT concentration were established. There was a duplicated well for each well for study. The cells were cultured in an incubator at 37℃, 5% CO₂ for 72 hours. MTT (5mg/ml) 10μl was added into each well 4 hours before the end of the experiment. After the crystalline dissolved completely, the absorption (OD) for each well was measured with enzyme labeling photometer at 570nm wave length and the 50% inhibitory concentration (IC₅₀) for each drug was calculated.

1.4 Assay of Cell Apoptosis with Terminal Deoxynucleotidyl Transferase (TUNEL) Method
In Situ Cell Death Detection Kits were used. 1×10⁵/ml cells were washed twice with phosphate buffer solution (PBS). 4% paraformaldehyde was added for fixation for 30 minutes. The suspension was centrifugalized at 2,000 rpm for 10 minutes. The supernatant was discarded. The cell sediment was washed with PBS again and 100μl 0.1% Triton-100 was added. The cells were chilled on ice for 2 minutes and washed with PBS one more time. To the cell sediment 50μl TUNEL labelling mixture (containing terminal deoxynucleotidyl transferase) was added. The cells were kept at 37℃ for 1 hour, washed twice with PBS and added with PBS 500μl. The cell suspension was ready for assay.

1.5 Assay of Cell Apoptosis with Annexin V Labeling Method
Annexin V Fluos reagent was used for labelling. 1×10⁵ cells were washed twice with Hank’s solution. Labeling buffer solution 100μl containing Annexin-V-Fluos 20μl and Propidium Iodide (PI) 20μl (50μl/ml) was added. The suspension was kept at room temperature for 15 minutes for assay.

2. Results

2.1 Inhibition of KLT on tumor cells
Inhibiting action on human erythroleukemia cells and solid tumor cells was observed after the cells were treated with KLT for 72 hours. The sensitivity (IC₅₀) of the tumor cells employed in this study to KLT was 38.5μl/ml for K562 cells, 22.6μl/ml for KB cells, 45.1μl/ml for SW1116 cells, 85.9μl/ml for WiDr cells, 21.4μl/ml for MKN28 cells and 185μl/ml for 7901 cells. The colo205 cells were relatively insensitive to KLT with an IC₅₀ >400μl/ml.

2.2 TUNEL technique for assay of KLT induced apoptosis of tumor cells
Fig.1 showed the apoptosis of human colon cancer SW 1116 cells after the cells were treated with KLT for 24 hours assayed by TUNEL method. When the cells were treated with 10μl/ml KLT the positive cell rate for the M2 area was 15.68% and that for the saline control group was 4.5%. When the cells were treated with 50μl/ml and 100μl/ml KLT respectively no marked
difference of positive cell percentage in the M2 area was observed between KLT treated groups and the saline control group, indicating that KLT at 10μl/ml concentration could induce apoptosis of SW 1116 cells.

2.3 KLT induced apoptosis of tumor cells assayed with Annexin V Labelling Method

Fig. 2 showed the apoptosis and necrosis of human erythroleukemia K562 cells after the cells were treated with KLT for 6 hours assayed by Annexin V Labeling Method. The M2 area of FL1-H indicated the Annexin V labelled positive cells. Only a few positive cells were found in the saline control group and emulsion (10μl/ml) group whereas the positive cells in the KLT (10μl/ml) treated group were almost doubled. The percentage of positive cells got decreased when the cells were treated with KLT at the concentration of 50μl/ml and 100μl/ml. The M3 area of FL2-H indicated PI high staining subgroup, suggesting the permeability of their cell membrane was markedly increased. The percentage of M3 positive cells of the saline control group and the emulsion group was very close but that of KLT (10μl/ml) treated group was doubled. When the cells were treated with 50μl/ml KLT the percentage of M3 positive cells was 40.7%, increasing almost to 4 times of the saline control group. When the cells were treated with 100μl/ml KLT the M3 positive cell rate was 28% -about 3 times of the saline control group.

3. Discussion

Apoptosis and necrosis are two different types of death for cells. Necrosis is a passive termination of the vital activity of cells, resulted from destroyed biomembrane and energy metabolism of the cells by external injury factors. Apoptosis is an active process of programmed death or suicide participated in by the cells. In apoptosis DNA chain of nucleosome is cut down by activated endonuclease and degraded into 200bp segments with a characteristic ladder appearance of electrophoretic pattern. Apoptosis can also be assayed by TUNEL technique. The phosphatidyl serine (PS) of normal cells is distributed at the inside of cell membrane but is exposed outside of the cell membrane during apoptosis. In the presence of Ca^{++}, PS can combine with Annexin V specifically. Therefore apoptosis can be probed by Annexin V. Living cells are resistant to PI staining. High staining of cells with PI indicates that the permeability of cell membrane is markedly increased. When Annexin V and PI staining are used together for assay, the hypochromasia of cells indicates the early stage of apoptosis and the hyperchromatic PI staining indicates cell necrosis or late stage of apoptosis.

Basic studies on KLT have revealed that the retardation of cell division in G2/M phase and the decrease of DNA synthesis (S-phase) by KLT accounted for the basic mechanism of its anticancer action. Retardation of cell division in G2/M phase was an important predisposing cause of apoptosis induced by many anticancer chemicals when they activated apoptotic factors in tumor cells. We have found that apoptosis could be induced in K562 cells after the cells were treated with KLT 10μl/ml for 6 hours and that injury of tumor cell membrane and necrosis of tumor cells as shown by PI high staining occurred with the increase of KLT concentration. The apoptosis of SW1116 cells had also been observed with TUNEL technique after the cells were treated with KLT 10μl/ml. But the percentage of TUNEL labelled positive cells got decreased with the increase of KLT concentration perhaps due to the increase of cell
necrosis. In this study obvious changes of the ultrastructure of tumor cells were also observed after the cells were treated by KLT, including wide vacuolar degeneration, chromatin condensation and apoptotic granules which were the morphological characteristics of cell apoptosis (See Fig.3).

4. Conclusions

In our experiment most tumor cells were sensitive to KLT with a few exceptions such as Colo205 cell strain. KLT induced apoptosis and necrosis of human tumor cells were the key mechanism for its killing action in the treatment of cancers and this action had a dose-effect relationship.

References

Fig1. Apoptosis of human colon cancer SW1116 Cells Induced by 24 Hour KLT treatment tested by TUNEL Method
A. Saline control Ggoup, B. KLT 10μl/ml, C.KLT 50μl/ml, D.KLT 100μl/ml
Fig 2. Apoptosis of human erythroleukemia K562 cells induced by 6 hour KLT treatment tested by Annexin V labeling.

A1-A5 Annexin V Labeling, B1-B5 PI Labeling
1. Saline Control    2. Emulsion 10 μl/ml    3. KLT 10 μl/ml    4. KLT 50 μl/ml    5. KLT 100 μl/ml